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Research Note—

Effect of Subtherapeutic Antimicrobials on Genetic Diversity of *Enterococcus faecium* from Chickens

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SUMMARY. The effect of growth promotants (bacitracin, virginiamycin, and flavomycin) on the genetic population of *Enterococcus faecium* isolated from a commercially integrated poultry farm was examined. A total of 551 *E. faecium* were isolated from chick boxliners ($n = 16$), litter ($n = 334$), feed ($n = 67$), and carcass rinse ($n = 134$) samples from four chicken houses. Two houses on the farm were control houses and did not use any antimicrobials while two other houses on the farm used flavomycin, virginiamycin, and bacitracin during six different chicken grow outs. BOX-PCR and pulsed-field gel electrophoresis (PFGE) results indicated that *E. faecium* strains had a high degree of genetic diversity as overall clustering was independent of source, house, or grow out. Similarity of ≥60% for the majority of BOX-PCR genogroups and ≥80% for the majority of PFGE genogroups was observed for a subset of carcass rinse samples ($n = 45$) examined. Seventy-nine percent (19/24) of isolates in BOX-PCR genogroup 2 also clustered in PFGE genogroup 2, although no association between the isolates and house or grow out was observed. These results suggest that *E. faecium* from chicken are genetically diverse and that growth-promoting antimicrobials do not affect the genetic population of *E. faecium*.

RESUMEN. *Nota de Investigación*—Efecto de dosis subterapéuticas de antimicrobianos en la diversidad genética de *Enterococcus faecium* en pollos.

Se examinó el efecto de promotores de crecimiento (bacitracina, virginiamicina y flavomicina) en la población genética de *Enterococcus faecium* aislados en granjas avícolas comerciales integradas. Se obtuvieron 551 aislamientos de *E. faecium* a partir de los fondos de cajas de pollitos ($n = 16$), cama ($n = 334$), alimento ($n = 67$) y de muestras de agua provenientes de canales lavadas de pollos provenientes de 4 galpones. Se emplearon 2 galpones como galpones control, en ausencia de antimicrobianos, mientras que en los otros 2 galpones se empleó flavomicina, virginiamicina y bacitracina durante la etapa de crecimiento de 6 lotes diferentes. Los resultados de las pruebas de reacción en cadena por la polimerasa de secuencias de elementos repetitivos y de electroforesis en campos eléctricos alternos indicaron que las cepas de *E. faecium* presentan un alto grado de diversidad genética ya que su clasificación fue independiente de la fuente, del galpón o del lote. Se observó una similitud mayor o igual al 60% y al 80% para la mayoría de los grupos genotípicos obtenidos mediante la prueba de reacción en cadena por la polimerasa de secuencias de elementos repetitivos y la prueba de electroforesis en campos eléctricos alternos, respectivamente, para un subgrupo de muestras de agua provenientes de canales lavadas ($n = 45$) examinadas. El 79% (19/24) de los aislamientos agrupados en el grupo genotípico 2 mediante la prueba de reacción en cadena por la polimerasa de secuencias de elementos repetitivos se localizaron igualmente en el grupo genotípico 2 mediante la técnica de electroforesis en campos eléctricos alternos, sin embargo, no se observó asociación alguna entre los aislamientos, galpones o lotes. Los resultados sugieren que las cepas de *E. faecium* en pollos son genéticamente diversas y que los antimicrobianos promotores del crecimiento no afectan la población genética del *E. faecium*.

Key words: antimicrobials, subtherapeutic, *E. faecium*, poultry, genetic diversity

Abbreviations: GPI = gram-positive identification card; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PFGE = pulsed-field gel electrophoresis; UPGMA = unweighted pair group method; USDA = U.S. Department of Agriculture; VREF = vancomycin-resistant *Enterococcus faecium*

The use of antimicrobials in food animal production has been the subject of much interest (4,14,16,25). In poultry production in the United States, antimicrobials are used therapeutically, subtherapeutically, and for nutritional or growth-promoting purposes. Growth-promoting antimicrobials improve both feed efficiency and weight gain in developing poults, although the mode of action during these processes is not well understood (8). Growth-promoting antimicrobial use in animals is considered a threat to human medicine because of the possibility of transferring antimicrobial-resistant normal microflora of animals via the food chain to humans and development of cross-resistance to therapeutic antimicrobial agents used to combat human pathogens (13,17). For example, recently Synercid, a streptogramin a and b antimicrobial, was approved for the treatment of vancomycin-resistant *Enterococcus faecium* (VREF). Because virginiamycin, an analog of Synercid, has been used in animal production for over two decades, it is possible that resistant *E. faecium* have already developed in the animal population (18).

The effect of growth-promotant usage on *E. faecium* antimicrobial susceptibilities in poultry has been examined previously; but to our knowledge, no study has investigated the effect of growth

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promotants on the genetic population of enterococci (3,5,6,22). In this study, the population of *E. faecium* on a poultry farm was examined by pulsed-field gel electrophoresis (PFGE) and BOX-polymerase chain reaction (PCR) to determine if usage of three growth promotants (flavomycin, virginiamycin, and bacitracin) caused a shift in genetic relatedness among *E. faecium* isolates. *E. faecium* isolates were collected from boxliner, litter, feed, and carcass rinse samples from a commercially integrated poultry farm before and after administration of growth promotants.

MATERIALS AND METHODS

Origin of samples. Between 2002 and 2003, samples were collected from a commercial four-house broiler farm in North Georgia. Broiler chickens from six grow outs from four different houses were studied. A grow-out period consisted of chickens ranging from a few days old to slaughter (8 wk old). The farm was contracted to raise chickens for a single commercially integrated company. The company provided the farm with the chickens and feed. Two houses were control houses (C) and two were treatment houses (T). Control houses received no antimicrobials, and chickens in those houses were not treated therapeutically with antimicrobials. The treatment houses were administered a different antibiotic for the six grow outs. The antibiotics used in the final feed before slaughter were flavomycin (20 g per ton) for grow-outs 1 and 5, virginiamycin (20 g per ton) for grow-outs 2, 3, and 4, and bacitracin (20 g per ton) for grow-out 6. Types of samples and methods of sampling and culturing were as follows.

Boxliners. Whole boxliners were collected after chicks were transported to the houses from the hatchery. Contents of the boxliners were sampled aseptically with swabs. Swab samples were placed into 50-ml conical tubes filled with 40 ml of 1× phosphate buffered saline (PBS, pH 7.2) and mixed with a shaker for 10 min. Debris was removed by filtering with gauze into a new conical tube and supernatant was discarded. One hundred microliters of the resulting pellet were removed for plating onto M-*Enterococcus* agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation.

Litter. Wood shavings from softwoods were used as bedding, material commonly used in poultry houses in the southeastern United States. The litter remained unchanged in each house throughout the study period. Litter samples were a composite of five locations in the house, which were then pooled. Five of these composite samples were collected from each house at weeks 4 and 7 per grow out. Five grams of chicken litter was weighed out in a 50-ml conical tube with 30 ml of 1× PBS (pH 7.2) and mixed with a wrist-action shaker for 5 min. Debris was removed by low-speed centrifugation (600 rpm, 15 min). The bacteria were pelleted by high-speed centrifugation (10,000 rpm, 15 min) and the supernatant was discarded. The resulting pellet was streaked onto M-*Enterococcus* agar (Becton Dickinson Microbiology Systems) for isolation and identification. Prelitter samples were composed of samples taken from the last layer of bedding used before the study was conducted.

Feed. Heat-treated pelleted feed was fed to the chickens *ad libitum.* All feed was stored in steel storage tanks with no access to rodents or wild birds. Control houses received feed without antibiotics, while treated houses received antibiotics as described above. Ten grams of feed were collected at 4 and 7 weeks per grow out. Samples were collected as the feed flowed from the pipes, which delivered feed to each feeder in the houses to eliminate contamination. Samples were taken aseptically, with changing of latex gloves between each sample. The samples were processed in the same manner as litter samples.

Carcass rinses. Ten chicken carcasses per house were randomly selected immediately before the chickens entered the cold-water chill tank and placed in a container with ice for refrigeration. Each whole chicken was rinsed in 250 ml of peptone water in an automated carcass shaker for 1 min at the Russell Research Center, U.S. Department of Agriculture (USDA), Athens, GA. Forty-five milliliters of rinsate from the bag were transferred to a 50-ml conical tube and 100 µl were inoculated on M-*Enterococcus* agar (Becton Dickinson Microbiology Systems) for isolation.

Isolation and initial identification. Ten well-isolated positive colonies from M-*Enterococcus* agar (Becton Dickinson Microbiology Systems) were subcultured onto blood agar and Enterococcosel agar (Becton Dickinson Microbiology Systems) and incubated for 24 hr at 37 C. Initial identification was performed using Gram staining, catalase test, bile-esculin test, and pyrrolidonyl-β-naphthylamide (7). One colony per isolate per house was selected and placed into a 96-well plate containing bile-esculin agar.

Identification of *Enterococcus* spp. by PCR and phenotypic testing. All isolates were tested in a multiplex, genus- and species-specific PCR of *Enterococcus* as previously described (10). Isolates were also screened using the automated Vitek 32 system (bioMerieux Vitek, Hazelwood, MO) Vitek Gram Positive Identification Card (GPI), according to the manufacturer's instructions.

BOX-PCR. BOX-PCR was performed as previously described with the following modifications (15). Whole-cell template was prepared by suspending a well-isolated colony in 50 µl ddH_20. The base master mix consisted of 5 µl of 20 mM $MgCl_2$ (with ficol and tartrazine) (Idaho Technology, Salt Lake City, UT), 2.5 µl of the BOXA2R primer (1.25 mM) (Operon, Alameda, CA), 0.5µl of a 10 mM dNTP mix (Roche, Indianapolis, IN), 2.5 µl of a 10% dimethyl sulfoxide (Sigma, St. Louis, MO), 11.5 µl of ddH_20, and 2.5 U *Taq* DNA polymerase (Roche). PCR reactions were performed in a final volume of 25 µl consisting of 22.5 µl of master mix and 2.5 µl of whole-cell template. Ten microliters of product was electrophoresed on a 1.5% 1× TAE agarose gel containing ethidium bromide. DNA molecular weight marker XVII (500 bp; Roche, Indianapolis, IN) was used as the standard. Electrophoretic separation was at 100 V for 85 min.

PFGE. Pulsed-field gel electrophoresis was performed as previously described (20). Briefly, cells from a 5-ml overnight culture were pelleted, embedded in agarose plugs, and lysed. Plugs were digested overnight with 20 U of *Sma*I (Roche) and digested DNA separated on a 1.2% SeaKem agarose gel using a CHEF-DRII pulsed-field electrophoresis system (BioRad, Hercules, CA). Electrophoresis was carried out at 6 V for 21 hr with a ramped pulse time of 5–30 sec in 0.5X Tris-borate-EDTA buffer (14°C).

Cluster analysis. Cluster analysis of PCR and PFGE results were determined using Bionumerics software program (Applied Maths, Sint-Martens-Latem, Belgium), using Dice coefficient and the unweighted pair group method (UPGMA). Optimization settings for both BOX-PCR and PFGE dendrograms were 1.06% and a band tolerance of 1%.

RESULTS

Genetic heterogeneity of poultry *E. faecium*. Cluster analysis was conducted on *E. faecium* isolates ($n = 551$) recovered from boxliners, feed, litter, and carcass rinsates to identify any genetic relatedness among sampling areas, houses (control *vs.* treated), and antimicrobial administered. Groups for BOX-PCR were defined as clusters having at least 60% similarity, while groups for PFGE had at least 80% similarity.

Results from both BOX-PCR analysis and PFGE indicated that poultry *E. faecium* were genetically heterogeneous, as distinct clustering could not be defined using any criteria tested. When *E. faecium* from all sampling areas were examined together, isolates were dispersed over the dendrogram in no discernible pattern, and groups had low similarity (data not shown). To determine if *E. faecium* from the same source would be more similar based on house or antimicrobial treatment, isolates from each source were analyzed separately. Although *E. faecium* from the same source had overall higher similarity ($\geq$60% similarity for BOX-PCR or $\geq$80% similarity by PFGE) than when all samples were examined together, clustering effects remained undetermined. An example of indiscriminate associations by sample is shown in BOX-PCR and PFGE analysis of a subset of carcass rinse samples (Fig. 1). Forty-five *E. faecium* from carcass rinsates were randomly selected from control

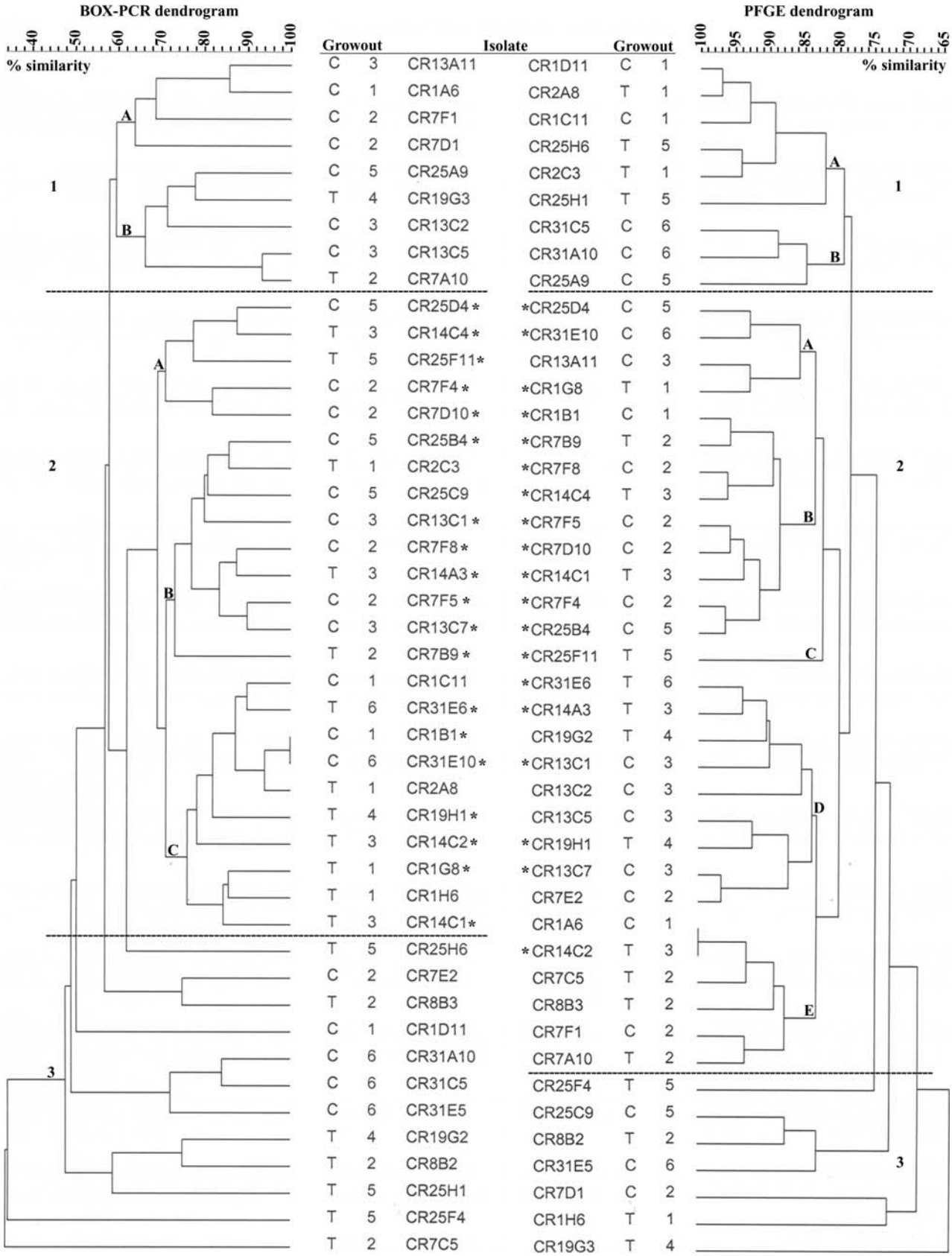

Fig. 1. Cluster analysis of *E. faecium* from poultry using BOX-PCR and PFGE. DNA for PFGE was digested with *Sma*I. No antimicrobial use (C) or treatment (T) with bacitracin (grow-out 6), flavomycin (grow-outs 1 and 5), or virginiamycin (grow-outs 2, 3, and 4) for each isolate is shown. Asterisks indicate *E. faecium* isolates common to both BOX-PCR and PFGE genogroup 2. Numbers and uppercase letters to the left and right of the dendrograms designate genogroups and subgenogroups, respectively. Levels of similarity were determined using Dice coefficient and UPGMA.

and treated houses and from each grow out and subjected to BOX-PCR and PFGE analysis. *E. faecium* from control houses were grouped with *E. faecium* from treated houses and from all grow outs, strongly suggesting that neither antimicrobial usage nor the antimicrobial had an effect on the population of *E. faecium* (Fig. 1).

Comparison of BOX-PCR and PFGE. To effectively examine the genetic relatedness of *E. faecium*, two different molecular-typing methods were compared. When a subset of *E. faecium* from carcass rinsates was analyzed using BOX-PCR and PFGE, two distinct clusters were formed by both methods (Fig. 1). BOX-PCR genogroups 1 and 2 were $\geq$62% and $\geq$70% similar, respectively, while PFGE genogroups 1 and 2 shared approximately 80% similarity. Various numbers of subgenogroups were identified for BOX-PCR and PFGE. Both BOX-PCR and PFGE genogroup 1 had the least number of subgenogroups (Fig. 1A,B), while PFGE genogroup 2 had five subgenogroups, the most of any genogroup examined (Fig. 1). A third genogroup for both BOX-PCR and PFGE was considered an outlier group, as a distinct cluster was not formed by the isolates (Fig. 1). Although genogroup 1 for both BOX-PCR and PFGE contained nine isolates each, genogroup 1 contained the least number of isolates for BOX-PCR while genogroup 3 contained the least number of *E. faecium* for PFGE. Only one *E. faecium* isolate in BOX-PCR genogroup 1 was also in PFGE genogroup 1. Three outlier *E. faecium* isolates in BOX-PCR genogroup 3 were also in PFGE genogroup 3 (Fig. 1). In contrast with genogroups 1 and 3, the majority of *E. faecium* grouped in genogroup 2 for both BOX-PCR and PFGE, and 79% (19/24) of *E. faecium* in BOX-PCR genogroup 2 were also in PFGE genogroup 2. These isolates were from a combination of control and treated houses and from different grow outs. Overall results from the two methods were comparable and indicate that related subpopulations exist within the sources of poultry *E. faecium*, although the common factor linking the isolates remained unidentified.

DISCUSSION

This study showed that the population of *E. faecium* from different poultry sources was not influenced by subtherapeutic doses of flavomycin, bacitracin, or virginiamycin. Clusters from individual sampling areas were examined based on whether they were administered growth promotant (treatment) or not (control), and the type of growth promotant received in the finisher diet. While *E. faecium* isolates were not tested for antimicrobial susceptibilities in this study, it was thought that isolates from control and treated houses would cluster separately due to antimicrobial usage. In previous studies, vancomycin-resistant *E. faecium* appeared to be more closely related than vancomycin-susceptible isolates and, in some cases, poultry isolates clustered separately from clinical and other animal *E. faecium* isolates (1,2,24). Results from this study indicated that neither antimicrobial treatment nor any other definitive factor examined influenced the genetic relatedness of the strains. This phenomenon has been observed previously in our laboratory when examining enterococci populations from poultry and swine in relation to antimicrobial resistance, farm, source, and species (11,12). Apparent links between enterococcal isolates and other traits could not be established.

Previous studies have shown that genetic heterogeneity among poultry *E. faecium* may be common, while other studies have found overall homogeneity among poultry *E. faecium*. In one study examining the effect of virginiamycin on antimicrobial resistance of *E. faecium* from poultry and swine, 17 different PFGE patterns were generated from 17 poultry *E. faecium*, suggesting genetic heterogeneity even in a small sample size and restricted environment. This is in contrast with another study that found that vancomycin-resistant and vancomycin-susceptible *E. faecium* from poultry were mostly a homogeneous population possibly due to preselection of a certain population during antimicrobial usage on poultry farms. In this study, distinct clusters of *E. faecium* were only apparent when subpopulations of isolates from the poultry sources were examined. *E. faecium* from this farm were genetically heterogeneous even after poultry were administered bacitracin, flavomycin, or virginiamycin during different grow-out periods. Subpopulations of more closely related *E. faecium* on the farm could be due to expansion of a clonal population that was only observed when the smaller sample size was examined or less genetic rearrangements between specific populations of poultry *E. faecium*.

PFGE is considered the gold standard for typing enterococci, but many other molecular techniques have been used to genotype enterococci as well (9,19,23). Although PFGE is a very useful technique for typing epidemiologically related strains, the procedure may misrepresent isolates that are unrelated epidemiologically. In addition, it has been previously stated that, to correctly determine genetic relationships among isolates, different typing methods should be used (21). Another typing method, BOX-PCR, is a relatively simple procedure to distinguish closely related organisms that is less expensive and faster than PFGE. For these reasons, PFGE was used along with BOX-PCR to fingerprint the poultry *E. faecium*.

Overall, both methods were successfully used in this study to fingerprint poultry *E. faecium*. The results with both BOX-PCR and PFGE analysis indicated a high degree of genetic diversity within poultry *E. faecium*, and there was no distinct clustering according to any criteria examined in individual dendrograms from all sources. Both procedures produced a range of band products and sizes that were comparable among the different sampling areas. In a previous report, BOX-PCR cluster results were comparable with those from PFGE. In this study, the similarities between isolates produced by each procedure correlated very well. Three clusters were generated for BOX-PCR and PFGE; genogroup 1 was composed of the same number of isolates, while genogroup 3 was composed of outlier isolates that did not have a high degree of similarity to the other two genogroups. In contrast, genogroup 2 was the largest cluster for both BOX-PCR and PFGE and 79% of the isolates in BOX-PCR genogroup 2 were also in PFGE genogroup 2. These results were very reassuring, as reproducibility in BOX-PCR is a major concern. One additional factor that favored comparable cluster analysis was the use of computational analysis for band comparison instead of visual analysis.

In conclusion, this study showed that subtherapeutic antimicrobial usage on a poultry farm did not alter the population of resident *E. faecium* from different sources on the farm. Poultry *E. faecium* are a genetically heterogeneous group that may have a subpopulation of isolates that have higher similarity to each other than to the overall population. Additional studies are needed to discern the underlying factor(s) that contribute to genetic heterogeneity in some populations of poultry *E. faecium*.

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